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Effects Of Osteoporosis Induced By Bilateral Ovariectomy On Estrogen Receptor Alpha And Beta Gene Expression And Hormonal Profiles In Rabbits.

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Abstract */ Osteoporosis is a serious health issue for older postmenopausal women. For the majority of postmenopausal women with osteoporosis, bone loss is associated with estrogen deficiency. This study aimed to investigate the role of estrogen α and β receptors in osteoporosis development in ovariectomized rabbits, with a focus on their correlation with bone loss. Twenty female rabbits with ovariectomies were split into two groups at random: the ovariectomized group (OVX group) and the control group. X-rays of the control and OVX groups were taken six weeks post-ovariectomy. In OVX models, the X-ray results showed a marked reduction in femoral fragility and bone density. Additionally, the results of gene expression show that alpha and beta estrogen receptors "Downregulation of ESR1/ERα in ovariectomized rabbits.: Research on postmenopausal individuals or animal models undergoing ovariectomies has demonstrated that estrogen deprivation may result in a decrease in ERα expression in bone cells. "However, ovariectomy led to upregulation of beta estrogen receptors (ESR2/ERβ) in bone cells, likely due to altered hormonal signals post-osteoporosis."*

Keywords: *Osteoporosis, Ovariectomy, Gene Expression, Estrogen Receptors , Rabbits.*

INTRODUCTION

Bone mass or bone density can be used to measure osteoporosis, a disease that affects the bones and is characterized by decreased bone mass, deterioration of bone structure, increased bone fragility, and an increased risk of fracture (**Aibar-Almazán *et al.,* 2022**) Osteoporosis reduces quality of life and daily functioning due to heightened fracture risk in areas such as the wrist, hip, and vertebrae (**Fitzgerald *et al* .,2024**).A number of factors, including natural and surgical ones, can cause a decrease in estrogen hormone, a crucial drug in the deposition of minerals in the tissues (bone mineralization) (**Aibar-Almazán *et al.,* 2022).** Different Methods for Creating Premature Ovarian Failure Animal Models Presently, the most prevalent animal models of premature ovarian failure include those resulting from chemotherapy drugs, radiation therapy, genetic induction, D-galactose, natural ovarian aging, oophorectomy, 4-vinylcyclohexene diepoxide, autoimmunity, psychological stress, and POF (**Pouladvand *et al.,* 2024 ;Baseem *et al* .,2024).).** Our rabbit model is a valuable tool to study osteoporosis (OP) because rabbits have much faster bone turnover than rodents or primates, and in contrast to rodents, they reach skeletal maturity soon after their sexual development is complete (**Gilsanz V *et al* ., 1988).**

Estrogen is a key hormone involved in the development and homeostasis of bone tissue in both males and females. Estradiol is the most potent estrogenic hormone in the human body. Estrogen action is controlled by two main estrogen receptors (ER), alpha and beta (ERα and ERβ), encoded by *ESR1* and *ESR2*, respectively. It regulates gene expression, metabolism, cell growth, and proliferation by acting through cytoplasmic signaling pathways or activating transcription in the nucleus. Estrogens bind to their receptors in the nucleus, acting as transcription factors regulatin ` g the expression of target genes. Estrogens can also

bind to their receptors outside of the nucleus activating signaling pathways in the cytoplasm. The cytoplasmic signaling pathway is activated by estrogen and growth factors and acts though the kinase signaling cascade which phosphorylates substrate proteins and transcription factors(**Manolagas *et al.*, 2013; Faltas *et al.*, 2020).** The primary source of cathepsin K (Cat K), which degrades collagen and other matrix proteins during bone resorption, is activated osteoclasts **(Costa ,2011)**.Deterioration of cathepsin K has the following effects: The proteolytic action of cathepsin K on the bone matrix degrades bone structure and decreases bone density, leading to osteoporosis. This increases the risk of fractures and bone deterioration. Serum Cat K levels were increased in postmenopausal osteoporosis patients. Moreover, the cysteine protease cathepsin K, which breaks down the proteins that comprise the bone matrix, facilitates osteoclast-mediated bone resorption **(Moon, 2024).** Increased in bone marrow adipose tissue (BMAT) in HFD mice has been reported previously in male mice **(Scheller *et al.*, 2016; Tencerova *et al.*, 2018)** while estrogen deficiency has been reported to increase BMAT in mice **(Elbaz et al., 2009; Georgiou *et al.*, 2012)** and humans (Veldhuis-Vlug & Rosen, 2018).Follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are produced by the anterior region of the pituitary gland, are referred to as gonadotropins **(Sadiq& Tadi (2023).** Because they regulate the two primary gonad functions—gamete formation and sex steroids (testosterone and estradiol)—both hormones are necessary for gonadal function in both men and women **(Al-Suhaimi *et al* ., 2022).**

Aim of study :

The present study aims to investigate the impact of bilateral ovariectomy in the rabbits for the depletion of estrogen levels on the following:-

1-Serum progesterone, estrogen and LH, and FSH levels
2-Bone biomarkers Cathepsin-K
3-Gene expression of estrogen alpha ESR1/ERα and beta receptors ESR2/ERβ
4-Detection bone loss by Xray

MATERIALS AND METHODS

Experimental Animals

Twenty 4-month-old female New Zealand white rabbits (weight: 800–850 g) were randomly divided into two groups (n = 10 per group) : a control group and bilateral ovariectomy (OVX)group. housed in wooden cages with ideal food, water, and ambient conditions for rabbit rearing were the study's participants. A 12-hour light-dark cycle, a relative humidity of 50 ± 5%, and a constant temperature of 25 ± 2 °C. They were divided into two groups and fed second-stage broiler feed, which had 3200 **(kcal/kg)**of energy, crude protein (19%), crude fat (4%), and fiber (≤1.9%)

1. The first group control group consists of Ten healthy female rabbits.
2 Ten healthy female rabbits in the second group bilateral ovariectomy (OVX undergo surgery to remove both of their ovaries, and they are treated with antibiotics for five days after the procedure.

Surgical Removal

Animals were prepared for surgery under sterile conditions, Animals were anesthetized with ketamine (40 mg/kg) and xylazine (10 mg/kg).For 30 minutes. A 4 cm incision was made in the skin extending from the umbilicus posteriorly. The incision was then extended to include the tunica albuginea and peritoneum after pushing the cecum aside to expose the uterus. By following the uterus upward from the left side, the left ovary could be accessed. Two ligatures were placed around the ovarian arteries above the ovary in the ovarian mesentery and two below the ovary in the cut ligament using 4/0 Catgut sutures. The suture between the two ligatures was then cut, and the suspensory ligament of the ovary was cut to free the ovary. The remaining blood vessels were examined for hemorrhage. The sutures were then tied together, and the operation was repeated on the right ovary. The abdominal wall was then closed with continuous sutures using 4/0 sutures, and the skin was closed with interrupted sutures using 4/0 silk sutures. The animals

were then cared for by sterilizing the wound and giving antibiotics for 5 days. The stitches were removed after 10 days (Silva and Coates, 2020).

Collection of Blood Samples.

The animals are anesthetized with ketamine and xylazine to control and relax them before the blood is drawn six weeks later. The blood is collected directly from the heart puncture. For the purpose of clotting, blood samples were left in non-heparin tubes at room temperature for 30 minutes. After that, the tubes were centrifuged for 15 minutes at 3,000 rpm to extract the serum, which was then taken out and put in fresh tubes, which were then kept in a freezer until they were needed. And Measure the levels of progesterone, estrogen, and FSH and LH. Use the biomarker ELISA (cathepsin ELK bioticnology China). Measure the genes ESR1/ERα and ESR2/ERβ (qRT-PCR . Examine the impact on bone mass.

Quantitative Reverse Transcriptase Real-Time Pcr

Animals were euthanized under anesthesia to collect samples (femoral heads). Thermofisher Scientific's TRIzolTM Reagent was used to preserve the organs in sterile, dark containers. The first strand of cDNA is made using a Roche or Promega first strand cDNA synthesis kit. The primer mixture consists of a random hexamer primer and an anchored oligo(dT)18 primer. To remove the traces of genomic DNA from the eluted total RNA using samples (DNase enzyme), the extracted total RNA was treated with DNase enzyme in accordance with the protocol described by the company **(Prasanthi *et al,* 2011)** Following cDNA synthesis, The expression of the target genes was quantified using gene-specific primers and 20µl reactions. Prepare the GoTaq 1-Step RT-qPCR Reaction Mix (total RNA 10 ml, Oligo(dT) 15 primer 10 pmole 2 ml, DEPC water 8 ml) by combining the standard materials, such as the Go TaqR 1-Step RT-qPCR Master Mix, CXR dye, nuclease-free water, and Go Script RT Mix, into a single batch. The thermal conditions were applied to all genes (Denaturation= 95 °C 20 sec-Annealing/Extension=60 °C 30 sec). Add the remaining ingredients after separating the mixture into different portions. The mRNA expression level of ESR1 and ESR2 bone resorption cytokines was measured by analyzing the relative quantities of glyceraldehyde 3-phosphate dehydrogenase mRNA. The methods described by **(Prasanthi *et al,* 2011)** were used for real-time PCR, cDNA synthesis, and RNA extraction. Table (1) contains a list of the primer sequences used in this study.

Ethical Approve

Under the reference number UOK.VET. PH.2024.095 ،the study was conducted at the Karbala University, College of Veterinary Medicines' anatomical facility in Iraq.

Statistical analysis:

Statistical analysis of data for experiments in the present study was performed by prism V8.0 on the basis of one way and two way analysis of variance (ANOVA) using significant level of ($P<0.05$) **(Buthelezi, 2024).**

RESULTS AND DISCUSSION:

1- Bone Density :

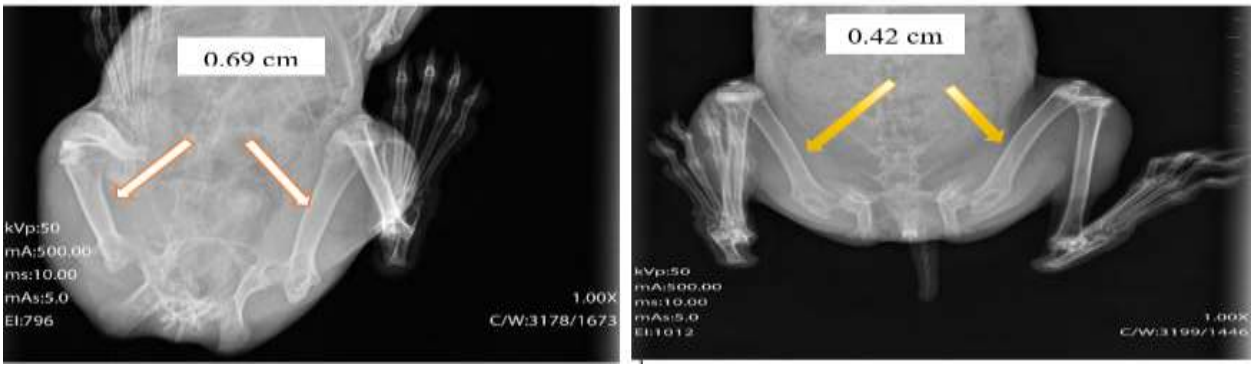

Figure 1:Radiographic image of the bones with white arrow of control group showing normal bone density (A). While Radiographic image of the bones with ovariectomy group (B) show a reduced bone density as camper with control group (after six month).

Effect Of Ovareictomy On Femoral Bone X-Ray

X-rays provide important information on the effect of reduced estrogen on osteoporosis. Compared to the control group, which has normal bones with the proper density, the OVX group's bones are less dense. X-rays are used to demonstrate the likelihood of osteoporosis. Loss of bone mass and an elevated risk of fractures and falls are the results of osteoporosis. Millions of people globally are impacted by this serious health issue This is consistent with our results **(Carey *et al* ., 2022 ;Balla *et al.*, 2019).**

2- Hormonal level:

A-Estrogen level :

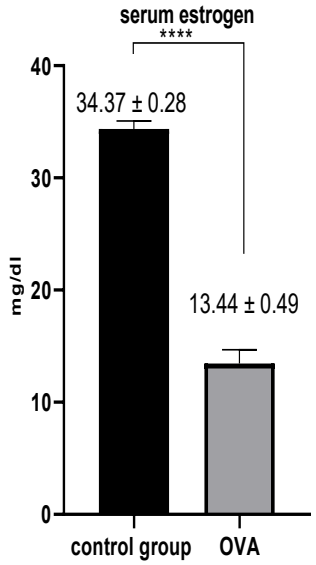

Figure 2: Estrogen serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group , and ovariectomized.

The current study showed a decrease in serum estrogen in the ovariectomized rabbit group compared to the control group, with the OVX group at 13.44 ± 0.49 versus 34.37 ± 0.28 in controls. The researchers reached a conclusion similar to what we have reached (**Iwasaki *et al* ., 2025 ;Coates& Silva ,2020 ; Nakata *et al* ., 2022**) . A lack of estrogen causes osteoclasts to increase and osteoblasts to decrease, leading to overall bone resorption. To lessen the nuclear factor-KB ligand's function By binding to the estrogen receptor and activating RANKL and osteoproteogerin (OPG), estrogen inhibits the growth of osteoclasts **(Cheng *et al.*, 2022)**. Estrogen receptors are expressed by osteoblasts, osteocytes, and osteoclasts. Additionally, estrogen directly influences bone through cytokines and local growth factors. The cause of osteoporosis is estrogen's suppression of IL-6 release, which encourages the recruitment of osteoclasts **(Alrowaili *et al.*, 2021)**. The activator of the nuclear factor-kappa B ligand receptor The RANKL/receptor activator of nuclear factor-kappa B (RANK) osteoprotegerin (OPG) system is the final typical mechanism for bone resorption. Osteoclasts and osteoclast precursors express RANK, which RANKL binds to to promote osteoclast development. OPG is a soluble decoy receptor that suppresses RANK-RANKL by binding and sequestering RANKL **(Tanaka, 2019; Cheng *et al.*, 2022)**.

B-Progesterone level:

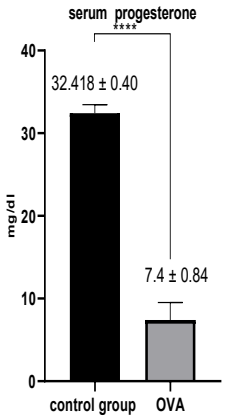

Figure 3 : progesterone serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group , and ovariectomized.

The current study showed a decrease in serum progesterone in the ovariectomized rabbit group compared to the control group, as the OVX group was 7.4±0.84 compared to the control group. 32.418±0.40 This is consistent with our results (**Tunheim, 2022 ; Hart ,2023**) . Progesterone, a steroid sex hormone produced by the ovaries, is crucial for embryogenesis, the menstrual cycle, and pregnancy. It is directly regulated by osteoblast progesterone receptors **(Al-Suhaimi *et al.*, 2022) .**These are present in both osteoblasts and osteoclasts. are increased by estrogen levels, which may suggest that progestogens are partially regulating the effects of estrogens on bone **(Mills *et al.*, 2021).**It has been reported that progesterone not only directly affects osteoblasts but also indirectly slows bone resorption through metalloproteinases and glucocorticoid receptors **(Wang *et al* ., 2023 ; Sharifi, 2023 ; Banoriya *et al* .,2025)**

C-LH level:

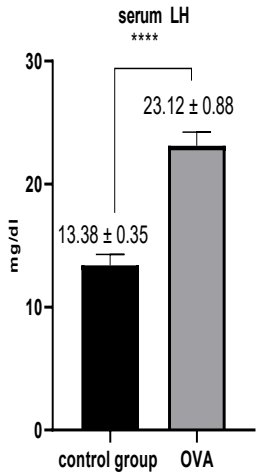

Figure 4 : LH serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group , and ovariectomized.

The current study showed an increase in serum LH hormone in the ovariectomized rabbit group compared to the control group, where the OVX group was23.12 ±0.88 compared to the control group. 13.38 ±0.35 .

We used OVX as a model for estrogen insufficiency in this study; however OVX also results in low levels of estradiol and high levels of gonadotrophins (FSH and LH). The elevation in bone marrow adipose tissue (BMAT) following OVX is probably caused by either a drop in estradiol or an increase in FSH. The researchers reached a conclusion similar to what we have reached either (**Liu *et al*.,** (2017) or both. The synthesis of RANKL by adipocytes may also be influenced by high FSH levels (**Juel Mortensen *et al.*, 2019**), nevertheless, the information currently available suggests that secreted RANKL has minimal impact on bone turnover or volume (**Juel Mortensen *et al.*, 2019**).

D- FSH level:

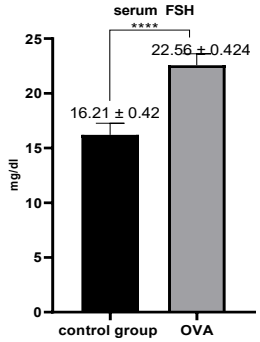

Figure 5 : FSH serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group , and ovariectomized.

The current study showed an increase in serum FSH hormone in the ovariectomized rabbit group compared to the control group, where the OVX group was 22.56±0.424 compared to the control group16.21±0.42. This is consistent with our results (**Alasmi, 2022;Zaidi *et al*., 2018**) Direct effects of FSH on bone cells. Osteoclast precursors express higher RANK and create more TNFα in response to FSH. It also enhanced the mechanisms that result in osteoclast differentiation (**Chin, 2018**).

E-Cathepsin K level:

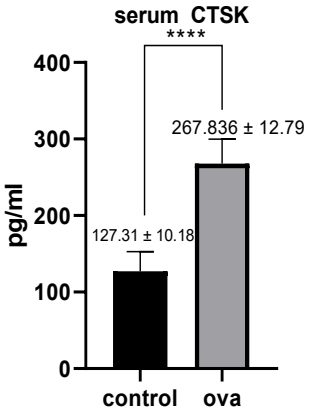

Figure 6 : Cathepsin K serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group , and ovariectomized

The current study showed an increase in serum levels in the OVX group compared to the control group Cathepsin K This is what the researcher agreed upon **(Demeuse *et al*., 2023)** Following ovarian excision, elevated cathepsin K is mostly caused by estrogen deprivation (**Al-khafaj *et al*., 2023)** .Osteoclasts are the cells that break down bone, and estrogen is crucial in preventing their activity(**Langton& Njeh, (2016).** Estrogen levels sharply decline following ovarian excision, which causes an increase in osteoclast activity and creation, elevated release of osteolytic enzymes, including cathepsin K, a protease enzyme required for the degradation of bone's type I collagen. **(Zhao *et al*., 2024)** One of the primary enzymes that osteoclasts use to break down bone matrix is cathepsin K, because bones are more prone to breaking down when estrogen levels are low, more cathepsin K is released (**Lewis, (2022) .** Type I collagen is broken down via osteoclast-driven bone resorption, which is mediated by cathepsin K. (**Shariati *et al*., 2025**). It is now believed that the primary enzyme responsible for degrading the organic bone matrix is cathepsin K, a cysteine protease belonging to the papain family. It is highly and specifically expressed in osteoclasts and possesses the unique ability to degrade type I collagen helical sections in acidic conditions , In contrast to the other cathepsins, osteoclasts were shown to express Cat K in high amounts **(Bautista-Carbajal *et al*., 2023).**

3-Gene Expression

Table 1 : Primers that were used in this study .

Primer	Sequence		Product size
Estrogen Receptor α rabbit	F	5-CATCCTCCTCCTCCTTGTGG-3	88 bq
	R	5-CAAAGGGTTCCTCGGAGACTG-3	
Estrogen Receptor Beta rabbit	F	5-CTCGATGTTTCCTTGGATGGTCCT-3	95 bq
	R	5-ATTGCCTCCGGCTACCACTAC-3	

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	45
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		
Melting curve	60-95°C	1

Table 2 : Downregulation of ERα in Ovariectomy Group Ct Values for ERα

Rabbit ID	Ct (ERα)	Ct (Housekeeping)	ΔCt (ERα)
Ovariectomy 1	28.5	22.0	28.5−22.0=6.5
Ovariectomy 2	29.0	21.8	29.0−21.8=7.2
Ovariectomy 3	27.8	22.3	27.8−22.3=5.5
Ovariectomy 4	28.2	22.1	28.2−22.1=6.1
Average ΔCt			(6.5+7.2+5.5+6.1)/4 =6.3

Rabbit ID	Ct (ERα)	Ct (Housekeeping)	ΔCt (ERα)
Control 1	25.0	22.0	25.0−22.0=3.0
Control 2	25.5	22.2	25.5−22.2=3.3
Control 3	25.3	22.1	25.3−22.1=3.2
Control 4	24.8	22.0	24.8−22.0=2.8
Average ΔCt			(3.0+3.3+3.2+2.8)/4 =3.1

significance = p-values > 0.05

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ΔΔCt and Relative Expression (ERα)

- **ΔΔCt: ΔCtOvariectomy−ΔCtControl=6.3−3.1=3.2**
- **Relative Expression: 2−3.2=0.11 (Downregulated in ovariectomy group)**
- **Livak formula 2- ΔΔCt**

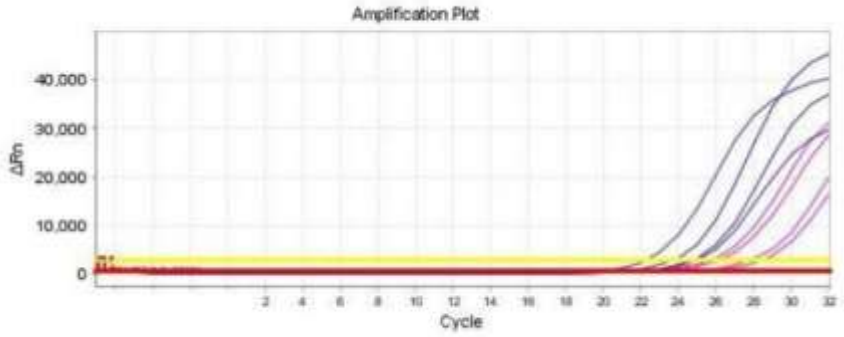

Figure 7 : Amplification curve of the tested samples represented the ERα gene. This indicate a successful RNA extraction and cDNA synthesis

Table 3 : Upregulation of ERβ in Ovariectomy Group Ct Values for ERβ

Rabbit ID	Ct (ERβ)	Ct (Housekeeping)	ΔCt (ERβ)
Ovariectomy 1	23.5	22	23.5−22.0=1.5
Ovariectomy 2	24	22.2	24.0−22.2=1.8
Ovariectomy 3	23.8	22.3	23.8−22.3=1.5
Ovariectomy 4	23.7	22.1	23.7−22.1=1.6
Average ΔCt			(1.5+1.8+1.5+1.6)/4 =1.6

Rabbit ID	Ct (ERβ)	Ct (Housekeeping)	ΔCt (ERβ)
Control 1	25.5	22	25.5−22.0=3.5
Control 2	25.8	22.1	25.8−22.1=3.7
Control 3	26	22.2	26.0−22.2=3.8
Control 4	25.9	22	25.9−22.0=3.9
Average ΔCt			(3.5+3.7+3.8+3.9)/4 =3.725

significance = p-values > 0.05

ΔΔCt and Relative Expression (ERβ)

- **ΔΔCt: ΔCtOvariectomy−ΔCtControl=1.6−3.725=-2.125**
- **Relative Expression: 2−(-2.125)=4.36**

- **(Upregulated in ovariectomy group)**
- **Livak formula 2- ΔΔCt**

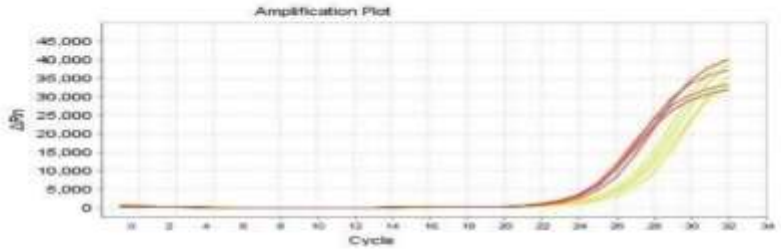

Figure 8 : amplification curve of the tested samples represented the ERβ gene. This indicate a successful RNA extraction and cDNA synthesis

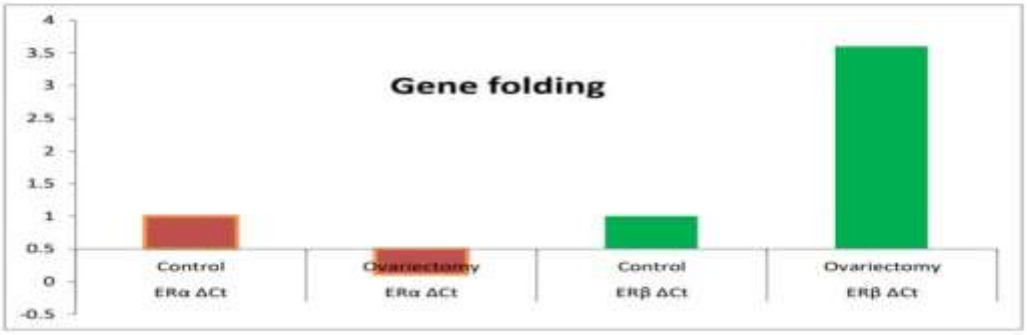

Figure 9: fold change comparison between the group expressed ERα and ERβ gene**.**

The current study demonstrated that there was a significant downregulation in the alpha estrogen receptor gene expression after 6 weeks in the control group (Average ΔCt 3.1) when compared to the group of rats who had their ovaries removed (Average ΔCt 6.3). These findings align with prior studies **(Cheng et al., 2022; Jin et al., 2015 ; Martin - Millan *et al.*, 2010)** .Downregulation: In postmenopausal people or ovariectomized animal models, ESR1/ERα expression in bone cells may be reduced when estrogen deficiency is present. By contributing to an imbalance in osteoclast and osteoblast activity, this decline exacerbates bone loss. **(Norton *et al.*, 2022).** According to the current study, the average ΔCt for beta estrogen receptor gene expression after six weeks was 3.725 in the control groups and 1.6 in the animals who had their ovaries removed (upregulated in the ovariectomy group). Beta estrogen receptors ESR2/ERβ in bone cells increased as a result of hormonal signal alterations after osteoporosis. The researchers came to the same result as us **(Balla *et al.* 2019; Shi & Morgan 2024).** In response to estrogen deprivation, bone shows elevated expression of the estrogen receptor beta (ESR2/ERβ) after ovarian excision. However, this increase is not enough to arrest bone loss because estrogen receptor alpha (ESR1/ERα) is essential for bone protection **(Khalid & Krum 2016; Mohanty *et al.*, 2025).** It has also been shown that estrogen and ERs affect the susceptibility of bone cells to mechanical loading and the subsequent bone cell mechanotransduction, which is the activation of fundamental mechanosensitive bone remodeling pathways **(Steppe *et al.*, 2021).** A previous analysis concentrated on the effects of

estrogen deficiency in osteocyte mechanobiology, but previous reviews have looked at the role of estrogen and ERs on bone biology and health in general **(Price *et al.*, 2011; Naqvi *et al.*, 2020).**

CONCLUSION:

Targeting ERα and ERβ agonists for postmenopausal osteoporosis therapy requires further investigation into their distinct roles in bone anabolism and resorption.

Recommendations:

1. Given the importance and influence of estrogen in treating postmenopausal osteoporosis, we recommend conducting a study on the potential of treating osteoporosis after menopause using estrogen, balancing luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the biological impact of this on various body organs.
2. Conduct further studies on another animal model to examine the physiological changes occurring in the body, to demonstrate the extent to which the effects differ between animals and those more closely related to humans.
3. The study demonstrated a significant impact on bone structure after menopause. Therefore, the study recommends studying the physiological changes in other body organs after menopause and examining the impact on each organ.
4. Suggest specific animal models (e.g., rodents vs. larger mammals) for future studies.

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NOVELTY STATEMENT: The importance of this study lies in its focus on the effect of estrogen deficiency on the body and the extent of its role in osteoporosis. This study can help identify the causes of osteoporosis in postmenopausal women.

AUTHORS' CONTRIBUTIONS: There are four authors contributed equally.

COMPETITION OF INTEREST: The authors declare no conflict of interest.

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